Active Nercc1 Protein Kinase Concentrates at Centrosomes Early in Mitosis and Is Necessary for Proper Spindle Assembly

Joan Roig,* Aaron Groen,[†] Jennifer Caldwell,[‡] and Joseph Avruch*

*Department of Molecular Biology and Medical Services, Massachusetts General Hospital and Department of Medicine, Harvard Medical School, Boston, MA 02114; [†]Department of Systems Biology, Harvard Medical School, Boston, MA 02115; and [‡]MDS Proteomics, Charlottesville, VA 22911

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The Nercc1 protein kinase autoactivates *in vitro* and is activated *in vivo* during mitosis. Autoactivation *in vitro* requires phosphorylation of the activation loop at threonine 210. Mitotic activation of Nercc1 in mammalian cells is accompanied by Thr210 phosphorylation and involves a small fraction of total Nercc1. Mammalian Nercc1 coimmunoprecipitates γ -tubulin and the activated Nercc1 polypeptides localize to the centrosomes and spindle poles during early mitosis, suggesting that active Nercc has important functions at the microtubular organizing center during cell division. To test this hypothesis, we characterized the *Xenopus* Nercc1 orthologue (XNercc). XNercc endogenous to meiotic egg extracts coprecipitates a multiprotein complex that contains γ -tubulin and several components of the γ -tubulin ring complex and localizes to the poles of spindles formed *in vitro*. Reciprocally, immunoprecipitates of the γ -tubulin ring complex polypeptide Xgrip109 contain XNercc. Immunodepletion of XNercc from egg extracts results in delayed spindle assembly, fewer bipolar spindles, and the appearance of aberrant microtubule structures, aberrations corrected by addition of purified recombinant XNercc. XNercc immunodepletion also slows aster assembly induced by Ran-GTP, producing Ran-asters of abnormal size and morphology. Thus, Nercc1 contributes to both the centrosomal and the chromatin/Ran pathways that collaborate in the organization of a bipolar spindle.

INTRODUCTION

The Nek protein kinase subfamily is named after the NIMA protein kinase of *Aspergillus nidulans*. NIMA has long been known to be involved in the control of mitotic entry and progression, inasmuch as *nimA* mutants arrest in G₂ with high Cdc2 activity, and when allowed to enter mitosis by the additional mutation of an APC subunit (bimE7), generate aberrant spindle and nuclear envelope morphologies. Reciprocally, overexpression of NIMA in *Aspergillus* results in chromosome condensation (possibly through direct histone H3 phosphorylation), aberrant spindle formation, and pseudomitotic arrest (for reviews, see Nigg 2001; O'Connell *et al.*, 2003).

NIMA overexpression in vertebrate cells can induce some mitotic features (O'Connell *et al.*, 1994; Lu and Hunter, 1995), suggesting that among the mitotic regulators in such cells are protein kinases whose substrate specificity overlaps that of *Aspergillus* NIMA. In fact, the human genome contains at least 11 protein kinases called *Neks* whose catalytic domains are ~40% identical to NIMA (for reviews, see Nigg, 2001; O'Connell *et al.*, 2003). None of the *Neks* characterized thus

far exhibit the broad impact on mitotic regulation of *Aspergillus* NIMA, suggesting that the expansion of the Nek family may reflect a subdivision of the functions of NIMA among a divergent array of related kinases and perhaps the introduction of new responsibilities as well.

Nek2 is localized to the centrosome, exhibits high activity in S and G₂, and has been shown to be involved in premitotic centrosome disjunction (Fry 2002). Nek1 and Nek8, although unrelated in their long carboxy-terminal noncatalytic tails, are encoded by genes whose mutation has been identified as the cause of two similar forms of autosomal recessive murine polycystic kidney disease (PKD) (Upadhyaya *et al.*, 2000; Liu *et al.*, 2002). All genes identified thus far as responsible for PKD encode polypeptides that function in the nonmotile cilia of renal epithelia, suggesting that Nek1 and Nek8 are also involved in the formation or function of cilia. In support of this suggestion, Fa2p, a Nek orthologue in *Chlamydomonas* has been shown to be important for ciliary function (Mahjoub *et al.*, 2004), and we have observed at least partial localization of Nek1 and Nek8 to the basal body of several epithelial cell types (Lenz, Rapley, and Avruch, unpublished data). Nek11 is activated by DNA damage and has been suggested to participate in S-phase DNA damage response (Noguchi *et al.*, 2002), whereas little is known concerning the regulation or functions of Nek3, Nek4, Nek5, and Nek10.

Nercc1/Nek9 (Roig *et al.*, 2002) is an ~120-kDa polypeptide whose aminoterminal catalytic domain is followed by a domain homologous to RCC1, the exchange factor for the small G protein Ran (a feature shared by Nek8). The RCC1 domain of Nercc1 acts as an autoinhibitory domain through the direct binding to Nercc1 protein kinase domain. Inactive

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Address correspondence to: Joan Roig (roig@molbio.mgh.harvard.edu) or Joseph Avruch (avruch@helix.mgh.harvard.edu).

Abbreviations used: γ -TuRC, γ -tubulin ring complex; MS, mass spectrometry; CSF, cytotostatic factor.

during interphase, Nercc1 is phosphorylated, upshifted slightly on SDS-PAGE, and activated during mitosis. Nercc1 is a good substrate *in vitro* for p34^{Cdc2} and is able to bind Ran both *in vivo* and *in vitro*, although it is not clear whether or how these properties are related to Nercc1 regulation or function *in vivo*. Nek6 and Nek7 bind strongly to the carboxy-terminal tail of Nercc1 distal to the RCC domain (Roig *et al.*, 2002); moreover, Nek6, which is itself a mitotic kinase, can be directly phosphorylated and activated by Nercc1 *in vivo* and *in vitro* (Belham *et al.*, 2003). Interference with Nercc1 or Nek6 function impedes mitotic progression. Microinjection of anti-Nercc1 antibodies during prophase induces arrest in prometaphase with disorganized spindle structures and misaligned chromosomes, or abnormal mitoses resulting in aneuploidy (Roig *et al.*, 2002); moreover, expression of Nek6 kinase-deficient mutants, or RNA interference (RNAi)-induced depletion of Nek6 also interferes with mitotic progression (Yin *et al.*, 2003).

Herein, we demonstrate that activation of Nercc1 *in vitro* and during mitosis *in vivo* requires autophosphorylation at a specific site on the Nercc1 activation loop. Nercc1 is found to coprecipitate with γ -tubulin, and the activated form of Nercc1 is concentrated on the centrosomes during mitosis. The orthologue of Nercc1 in *Xenopus laevis* (XNercc) associates in egg extracts with the γ -tubulin ring complex (γ -TuRC), a multisubunit complex responsible for the microtubule nucleation activity of centrosomes (see, for example, Job *et al.*, 2003); moreover, XNercc localizes in part to the poles of spindles formed in *Xenopus* egg extract spindles. Immunodepletion of XNercc from cytosolic factor (CSF)-arrested egg extracts does not affect Ca²⁺-induced cell cycle progression, but it interferes with the correct formation of bipolar spindles; addition of recombinant XNercc corrects these abnormalities. Xnercc depletion also delays the formation of Ran asters, a process that is independent of centrosomes and physiologically directed by chromatin (Gruss and Vernos, 2004).

These findings point to a direct role for Nercc1 in the microtubule organizing function of centrosomes during mitosis as well as in the Ran-directed pathway of microtubule assembly. Nercc1 thus plays an important role in both the chromosomal and centrosomal pathways of microtubule polymerization that collectively result in the formation of the mitotic spindle.

MATERIALS AND METHODS

Mass Spectrometry (MS) Determination of P-Sites

Gel bands were excised as digested with trypsin, and resultant peptides were extracted from the gel. An aliquot of peptide was loaded onto a fused silica (360 μ m OD, 50 μ m ID; PolyMicro Technologies, Phoenix, AZ) capillary C18 (ODS) column with a pulled emitter tip (19). Peptides were high-performance liquid chromatography (HPLC) gradient eluted and analyzed by a LCQ DECA XP ion trap mass spectrometer (Thermo Electron, Waltham, MA). The mass spectrometer was set to data dependent mode to take tandem mass spectrometric (MS/MS) spectra of the top five most abundant *m/z* peaks in each MS scan. The MS/MS spectra were searched (Sequest; Thermo Electron) against a database containing the protein in order to deduce the sequences. Potential posttranslational modifications were also searched [STY = 80 (phosphorylation)]. An additional aliquot of each sample was subjected to immobilized metal affinity chromatography (IMAC) to enrich the sample for phosphorylated peptides. Briefly, peptides are loaded onto a capillary IMAC column. The column is washed to remove nonspecific binding and then the peptides are eluted onto a capillary C18 precolumn. The precolumn is washed with HPLC buffer and then connected to the analytical column described above. The peptides are eluted and analyzed as described above. Sequest peptide sequences were manually confirmed to ensure correct sequence identification.

Protein Identification by MS

Protein identification was performed at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA); after SDS-PAGE electrophoresis and Coomassie staining relevant bands were excised, subjected to tryptic digestion *in situ*, and extracellular peptides were separated and analyzed by microcapillary liquid chromatography/tandem mass spectrometry (LC/MS/MS), using an LCQ DECA ion-trap mass spectrometer (Thermo Electron).

Antibodies

To produce an anti-Nercc1 Thr210 phosphospecific antibody, synthetic peptides corresponding to human Nercc1 amino acids 205–215 plus a carboxy-terminal cysteine were synthesized containing either a phosphorylated or nonphosphorylated threonine at position 210. The phosphorylated peptide was coupled through the carboxy-terminal cysteine to KLH, and the conjugate was used to immunize rabbits at Cocalico Biologicals Affinity purification of peptide-specific IgG used the phosphorylated peptide coupled to SulfoLink columns (Pierce Chemical, Rockford, IL). Subsequently, a similar column with coupled unphosphorylated peptide was used to remove antibodies that could recognize unphosphorylated Nercc1.

Anti-XNercc antibodies were produced similarly using peptides corresponding to the *Xenopus* XNercc amino acids 3–18 plus a carboxy-terminal cysteine (anti-N-XNercc) and 810–825 plus an aminoterminal cysteine (anti-C-XNercc).

Rabbit Xgrip109 antibodies were generated against a fusion between glutathione *S*-transferase (GST) and amino acids 134–244 of Xgrip109 as described previously (Martin *et al.*, 1998). The Xgrip109 fragment was PCR amplified using an Xgrip109 cDNA as template (ACTT-LGC Promochem, Teddington, United Kingdom) with a cloning strategy similar to that described previously (Martin *et al.*, 1998). GST-Xgrip109 protein was expressed and purified using conventional methods (GE Healthcare, Piscataway, NJ). Xgrip109 antibodies were specifically affinity purified from GST-depleted serum and the specificity of antibodies was confirmed by Western blot analysis.

Anti- γ -tubulin and anti-FLAG antibodies were from Sigma-Aldrich (St. Louis, MO). Fluorophore or horseradish peroxidase-conjugated secondary antibodies were from Sigma-Aldrich or Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture and Immunotechniques

Cell culture, lysis, immunoprecipitation, and immunoblotting methods have been described previously (Roig *et al.*, 2002). For immunohistochemistry, cells were grown on coverslips, rinsed with phosphate-buffered saline (PBS), and fixed by immersion in methanol at -20°C for 5–10 min. After rinsing with PBS, cells were incubated in solution A (3% bovine serum albumin in PBS plus 0.1% Triton X-100 and 0.02% azide) for 1 h. Cells were then incubated with different antibodies diluted at a final concentration of 1 $\mu\text{g}/\text{ml}$ (except anti- γ -tubulin, used at 1:2000) in solution A. To visualize primary antibodies, coverslips were incubated with labeled secondary antibodies with the appropriate fluorophore diluted in solution A: fluorescein-conjugated donkey anti-rabbit and rhodamine X-conjugated donkey anti-mouse. After repeated washes with PBS, cells were incubated with 0.01 mg/ml 4,6-diamidino-2-phenylindole (DAPI) to stain the DNA and mounted in a microscope slide.

cDNA Cloning

Using the BLAST program, multiple overlapping *Xenopus* expressed sequence tags (ESTs) corresponding to the genomic sequences of XNercc (Roig *et al.*, 2002) were identified, enabling the assembly of a predicted cDNA. This XNercc cDNA was cloned by PCR from an oocyte cDNA library (a gift from J. Maller, University of Colorado, Boulder, CO) and subcloned into pCMV5-FLAG vector.

Xenopus Egg Extracts

Metaphase-arrested *Xenopus* egg extract, X-rhodamine-labeled tubulin, demembrated sperm nuclei, and extract spindles were prepared as described previously (Lohka and Masui, 1984; Hyman *et al.*, 1991; Desai *et al.*, 1999). Cycled meiotic spindles assembled from sperm nuclei were formed as described previously (Desai *et al.*, 1999). RanGTP poles were induced by the addition of Ran(Q69L)GTP to 0.3 mg/ml CSF (Bischoff *et al.*, 1994; Stewart *et al.*, 1998).

XNercc-immunodepleted *Xenopus* egg extract was made by three rounds of incubating 50 μl of protein A Dynabeads (DynaL Biotech, Lake Success, NY) bound to 20 μg of affinity-purified XNercc polyclonal antibodies with 150 μl of extract for 1 h at 4°C . Immunoblots were used to confirm depletions. Cycled *Xenopus* meiotic spindles were fixed and stained for immunofluorescence as described previously (Desai *et al.*, 1999). Antibodies were used at concentrations of 1 $\mu\text{g}/\text{ml}$.

Figure 1. Phosphorylation of Nercc1 kinase (Thr210) during autoactivation in vitro and during mitosis. (A) Autoactivation of recombinant Nercc1 in vitro requires Thr210. Wild-type and T210A mutant forms of Nercc1 were expressed in HEK293T cells, immunoprecipitated using FLAG antibody, washed, and incubated for the indicated times with phosphorylation buffer containing [γ - 32 P]ATP and model substrate histone H3. FLAG Western blots, and 32 P autoradiographs of XNercc and histone H3 are shown. (B) Specificity of an anti-phospho-Thr210-Nercc1 antibody. Recombinant FLAG-Nercc1 was expressed in HEK293T cells, immunoprecipitated using FLAG antibody, washed, and incubated in phosphorylation buffer with or without 100 μ M ATP. Western blots using FLAG antibody and purified anti-phospho-Thr210-Nercc1 (a-P-Nercc1) antibody are shown. (C) Anti-Nercc1 and anti-phospho-Thr210-Nercc1 immunoblot of anti-Nercc1 polypeptide immunoprecipitates. Immunoprecipitates prepared from extracts of exponentially growing (Exp) or nocodazole arrested, mitotic (M) U2OS cells using normal rabbit IgG (NlgG, lanes 1, 2, 7, and 8) or anti-total Nercc1 antibody raised against the N-terminal (a-Nercc1 [Nt], lanes 3, 4, 9, and 10) or the C-terminal tail (a-Nercc1 [Ct], lanes 5, 6, 11, and 12) of Nercc1, were subjected to immunoblot with anti-Nercc1 (Nt) polypeptide (lanes 1–6) and anti-phospho-Thr210-Nercc1 (lanes 7–12). (D) Anti-phospho-Thr210-Nercc1 immunoblot of anti-Nercc1 polypeptide immunoprecipitates. Immunoprecipitates from extracts of exponentially growing (Exp) or mitotic (M) U2OS cells are immunoblotted for Nercc1 polypeptide (lanes 1–4 and 9–12) or phospho-Thr210-Nercc1 (lanes 5–8). An overexposure of the blot in lanes 1–4 is shown in lanes 9–12 to make visible the high-molecular weight form of Nercc1, indicated by the asterisk, that is present in mitotic cells.

Recombinant XNercc Expression and Purification

XNercc was expressed as a FLAG-fusion protein in human embryonic kidney (HEK)293T cells. Cells were lysed as described previously (Roig *et al.*, 2002) and immunoprecipitated with anti-FLAG-Agarose (Sigma-Aldrich). After washing, FLAG-XNercc was eluted at room temperature with 0.1 mg/ml FLAG peptide (Sigma-Aldrich) in 10 mM HEPES, pH 7.7, 200 mM KCl, 10% sucrose, 1 mM dithiothreitol, plus protease inhibitors, and stored at -70°C until use. The purity of the FLAG-XNercc was evaluated by SDS-PAGE; in addition, γ -tubulin was not detectable by immunoblot of the FLAG-XNercc isolates.

RESULTS

An Anti-Phosphopeptide Antibody That Specifically Recognizes Active Nercc1

Nercc1 endogenous to mammalian cells is activated in vivo during mitosis; recombinant Nercc1 recovered after tran-

sient expression is inactive, but it can be activated in vitro by autophosphorylation, a reaction that requires Nercc1 homooligomerization, and is presumably carried out in *trans* within the Nercc1 dimer (Roig *et al.*, 2002). Reasoning that the mechanism underlying Nercc1 autoactivation in vitro is likely to participate in the process of Nercc1 mitotic activation, we used mass spectrometry (LC/MS/MS) to analyze the sites of phosphorylation on recombinant Nercc1, examined before and after autoactivation in vitro. A single phosphorylated residue, Thr333, was detected on inactive recombinant Nercc1, whereas Nercc1 autoactivated in vitro showed at least 12 additional phosphorylated serine and threonine residues. Of particular interest were the phosphorylation of Nercc1 Ser206 and Thr210, residues located on the activation loop of the protein kinase domain. We

Figure 2. Activated Nercc1 is concentrated at the centrosome and binds γ -tubulin. (A) Specificity of the anti-Nercc1 Thr210P immunoblot. An anti-phospho-Thr210-Nercc1 immunoblot of total cell extracts from exponentially growing (Exp) and mitotic U2OS cells (M, arrested by overnight incubation with nocodazole). (B) Immunolocalization of active Nercc1 in interphase U2OS cells using the anti-phospho-Thr210

previously observed that the homologous residues on the activation loop of the active form of Nek6 (Thr202 and Ser206) are phosphorylated, and the phosphorylation of Nek6 Ser206 is indispensable for Nek6 activity (Belham *et al.*, 2003). Nek6 Ser206 corresponds to Nercc1 Thr210, suggesting the likelihood that the phosphorylation of Nercc1 Thr210 is critical for Nercc1 activation. In fact, the Nercc1 mutant Thr210Ala is unable to catalyze any autophosphorylation in vitro on incubation with Mg^{2+} ATP and is entirely devoid of kinase activity toward exogenous substrates (Figure 1A). Thus, Nercc1 Thr210 is phosphorylated upon activation of the protein kinase in vitro, and this phosphorylation is necessary for Nercc1 activation.

We next sought to generate an anti-Nercc1 Thr210 phosphospecific antibody, so as to determine whether Nercc1 Thr210 undergoes phosphorylation during the mitotic activation of endogenous Nercc1. Rabbits were immunized with a phosphopeptide corresponding to the amino acid sequence surrounding human Nercc1 Thr210P; the purified, phosphopeptide-specific IgGs exhibited reactivity with recombinant Nercc1 only after the kinase had been autoactivated in vitro by incubation with Mg^{2+} ATP (Figure 1B). These results confirm that Thr210 is phosphorylated during activation in vitro and demonstrate the very high specificity of the anti-Thr210P antibody for the form of Nercc1 activated in vitro. Anti-Nercc1 immunoprecipitates were prepared from cycling and nocodazole-arrested, mitotic U2OS cells, using anti-Nercc1 antibodies raised against two different synthetic peptides corresponding to Nercc1 amino-terminal (Nt) or carboxy-terminal (Ct) sequences. Immunoprecipitates from cycling cells show a single band immunoblotable with either anti-Nercc1 antibody (Figure 1C, lanes 3 and 5; our unpublished data), whereas immunoprecipitates from mitotic cells occasionally show one or more additional minor but clearcut upshifted Nercc1 bands (Figure 1C, lanes 4 and 6). Immunoblot of these anti-Nercc1 immunoprecipitates with the anti Nercc1(Thr210P) antibody gives little or no signal in the samples prepared from cycling cells (Figure 1C, lanes 9 and 11; Figure 1D, lane 7), whereas immunoblot of the anti-Nercc1 immunoprecipitate from mitotic U2OS cells exhibits an array of immunoreactive bands, at M_r ranging from 120 to 170k Da (Figure 1C, lanes 10 and 12), that overlap with the minor upshifted band of Nercc1 immunoreactivity, but contain species migrating even more slowly than the minor band visible in the Nercc1 immunoblots (Figure 1C, compare lanes 4–10 and 6–12). In some experiments, the majority of Nercc1(Thr210P) immunoreactivity migrates as a dominant 170-kDa band (Figure 1D, lane 8) that corresponds to a faint band of immunoreactive Nercc1 (Figure 1D, lane 12, denoted by an asterisk). The selective appearance of the Nercc1(Thr210P)-immunoreactive bands in extracts of mitotic cells, a situation shown previously to correspond to the timing of Nercc1 kinase

activation, together with the specific precipitation of the Nercc1(Thr210P) immunoreactive bands by two independent anti-Nercc1 antibodies provides strong evidence the Nercc1(Thr210P)-immunoreactive bands represent Nercc1 polypeptides that have undergone mitotic activation involving autophosphorylation, as occurs in vitro. The relatively low fraction of total Nercc1 that participates in mitotic activation, illustrated most dramatically in Figure 1D, is surprising, but probably accounts for the finding that although Nercc1 can be readily activated ~100-fold in vitro, the apparent increase in Nercc1 kinase activity measurable in immunoprecipitates of total Nercc1 from mitotic cells is no more than approximately fivefold (Roig *et al.*, 2002). Moreover, the extent of slowing of the electrophoretic mobility of the Thr210-phosphorylated Nercc1 polypeptides is striking, and the basis for this phenomenon is not known. Immunoblots of Nercc1 immunoprecipitates from mitotic cells using anti-ubiquitin and anti-polyADP-ribose antibodies are negative, and although omission of phosphatase inhibitors (i.e., calyculin or NaF) from the extraction medium eliminates recovery of Nercc1Thr210P immunoreactive bands (our unpublished data), it is not clear whether the upshift is due entirely to Nercc1 phosphorylation per se.

Activated Nercc1 Is Specifically Localized to the Centrosomes and Mitotic Spindle Poles

Recombinant and endogenous Nercc1 polypeptide is diffusely distributed in the cytoplasm both during interphase and mitosis; a brief saponin treatment of mitotic cells released essentially all detectable immunoreactive Nercc1, in a manner similar to lactic dehydrogenase (Roig *et al.*, 2002). In view of the evidence suggesting that only a small fraction of Nercc1 undergoes activation during mitosis (Figure 1D), we attempted to use anti-Nercc1Thr210P antibody to track activated Nercc1 during the cell cycle. The validity of this approach is supported by the very specific immunoblots of whole cell lysates obtained using the anti-Nercc1(Thr210P) antibody (Figure 2A). When exponentially growing U2OS cells were stained with anti-Thr210P antibodies, only a small fraction of cells exhibited immunoreactivity that was clearly above background; in those cells, Nercc1Thr210P immunoreactivity was evident most clearly as one or two bright dots located close to the nucleus, and more weakly within the nucleus in a diffuse speckled pattern (Figure 2B, left, arrowheads). This staining was specifically inhibited by addition of the phosphorylated Nercc1Thr210P synthetic peptide used as antigen, whereas the corresponding nonphosphorylated peptide did not affect the observed pattern (Figure 2C).

Microinjection of several anti-Nercc1 polypeptide antibodies in prophase was shown previously to result in a high fraction of cells with disorganized mitotic spindles (Roig *et al.*, 2002). This response, together with the mono- or bipunctate perinuclear localization of activated Nercc1 suggested that active Nercc1 is concentrated in the centrosomes, organelles that provide a microtubule organizing function and are intimately involved in the assembly of the mitotic spindle in most animal cells (Doxsey, 2001; Rieder *et al.*, 2001). Costaining of cells with anti-Nercc1Thr210P and the centrosomal marker γ -tubulin, confirmed the localization of active Nercc1 to the centrosome (Figure 2, B and C). Staining of the centrosomes with the anti-Nercc1Thr210P antibody is independent of the fixation method, because it was observed either after methanol or glutaraldehyde/paraformaldehyde fixation. In addition to their colocalization, Nercc1 and γ -tubulin also exhibit a direct physical association; anti-Nercc1 polypeptide immunoprecipitates prepared from exponentially growing and mitotic U2OS cells specifically coprecipitate

Figure 2 (cont). Nercc1 antibodies. Costaining is with anti- γ -tubulin and DAPI (DNA). Bar, 15 μ m. (C) P-peptide competition of anti-phospho-Thr210 Nercc1 immunofluorescence. As in A, but the antibodies were preincubated either with no peptide (top row) or the immunizing peptide in its unphosphorylated (middle row) or phosphorylated (bottom row) form. For clarity, prometaphase cells are shown. Bar, 15 μ m. (D) Nercc1 coprecipitates γ -tubulin. Immunoprecipitates prepared from extracts of exponentially growing (Exp) or nocodazole arrested, mitotic (M) U2OS cells using normal rabbit IgG (NIgG), or anti-total Nercc1 antibody (a-Nercc1) were subjected to immunoblot with either anti-Nercc1 (top) or anti- γ -tubulin (bottom) antibodies.

Figure 3. Immunolocalization of active Nercc1 during mitotic progression in U2OS cells. Staining of active Nercc1, DNA, and γ -tubulin as described in A. Bar, 15 μ m.

tate γ -tubulin; somewhat higher amounts of γ -tubulin are consistently recovered in Nercc1 immunoprecipitates from mitotic cells (Figure 2D; see below).

We monitored Nercc1 activation in exponentially growing U2OS cells, stained in parallel with γ -tubulin and DAPI

(Figure 3). Interphase cells, identified by the presence of one or two closely spaced dots of γ -tubulin-staining indicating nonseparated centrosomes, show little or no P-Nercc1 signal. Cells with separated centrosomes and condensed DNA, and thus in prophase, show in all cases clearly positive

centrosomal staining for Nercc1Thr210P, and cells in metaphase and anaphase show even more strongly stained spindle poles (~3-fold stronger signal in metaphase than in prophase cells). A strong Nercc1Thr210P signal on the metaphase spindle poles was also observed when cells were arrested in metaphase by treatment with the proteasome inhibitor MG132 (our unpublished data). As cells entered anaphase, the Nercc1Thr210P signal becomes weaker at the poles and detectable on the chromosomes; Nercc1Thr210P immunoreactivity in the spindle poles/centrosomes decreases further during telophase and cytokinesis and essentially disappears in interphase cells. Cells that are going through cytokinesis show an additional phospho-Nercc1 signal at the midbody, composed of two strong spots surrounding the cytokinetic furrow.

In summary, during interphase, Nercc1 is found in a stable (i.e., immunoprecipitable) complex with γ -tubulin. Although the earliest time of Nercc1 activation remain uncertain, once activated in prophase, the protein kinase is strongly concentrated on the centrosomes and remains predominantly at the spindle poles during prometaphase/metaphase. The association of Nercc1 with γ -tubulin, one of the components critical for centrosomal nucleation of microtubules during mitosis, together with the ability of anti-Nercc1 antibody microinjection to interfere with spindle assembly, suggests a role for active Nercc1 in spindle assembly. In addition, a significant fraction of activated Nercc1 becomes associated with the chromosomes after the metaphase-to-anaphase transition, finally migrating to the midbody/spindle midzone. The relocalization of active Nercc1 onto the chromosomes and midbody raise the possibility that Nercc1 may have additional functions after the metaphase-to-anaphase transition.

Xenopus Nercc1 Function: Nercc1 in Frog Egg Extracts

We attempted to extend our studies of Nercc1 function in mammalian cells using specific small interfering RNA molecules; however, in contrast to the strong phenotypes observed with microinjection of anti-Nercc1 antibodies, RNAi-induced depletion of endogenous Nercc1 polypeptide by $\leq 80\%$ has not resulted in an observable phenotype; we presume that the remaining enzyme is sufficient to sustain physiological Nercc1 functions. We therefore explored the utility of *Xenopus laevis* egg extracts (Murray, 1991), a system widely used to study spindle formation and function (Desai *et al.*, 1999), as a model for further investigation of the role(s) of Nercc1 in spindle organization.

A search of *X. laevis* databases yielded DNA sequences that coded for polypeptides homologous to the human Nercc1 (Nercc1, accession no. AY080896). Several protein kinase domains corresponding to different members of the NIMA family were detected, including a *Xenopus* Nek6 and Nek7 (Roig and Avruch, unpublished data); however, only a single *Xenopus* sequence was identified from overlapping ESTs that contained the hallmarks of Nercc1, i.e., an NIMA-like protein kinase domain followed by several RCC1 repeats and a C-terminal extension containing a coiled-coil. No Nek8 homologues were detected (i.e., a NIMA-like kinase domain followed by RCC1 repeats but without the Nercc1 C-terminal extension). We isolated a cDNA encoding the predicted *Xenopus* Nercc1 sequence by PCR using an oocyte cDNA library (a gift from J. Maller) as template. The 2835 base pairs cDNA thereby obtained (accession no. AY271412) corresponded in DNA sequence to that predicted from the ESTs and encoded a polypeptide of 944 amino acids called XNercc (Figure 4A) that is 68% identical (79% similar) to the human Nercc1. The main differ-

ences between the *Xenopus* and human Nercc1 are in the N terminus of the proteins, upstream of the protein kinase domain, and in the region carboxy terminal to the RCC1 repeats and the poly-(Gly/Ser) rich-segment; importantly however, like human Nercc1, the *Xenopus* Nercc1 carboxy terminus contains a coiled-coil domain presumably important for homodimerization. Transient expression of a FLAG-XNercc1 in HEK293 cells yielded a 120-kDa polypeptide that exhibited autophosphorylation and autoactivation in vitro (Figure 4B), resulting, however, in an apparent kinase-specific activity well below that of human Nercc1. Unfortunately, the anti-Nercc1Thr210P antibody, created against the human Nercc1 sequence, showed no reactivity with recombinant (or endogenous; see below) XNercc1.

Xenopus XNercc Binds the γ -Tubulin Complex and Localizes to Spindle Poles

An antibody was raised against a synthetic peptide corresponding to XNercc amino acids 3–18 (referred to henceforth as anti-N-XNercc). This antibody efficiently immunoprecipitates recombinant XNercc1 expressed in mammalian cells, and on immunoblot of *Xenopus* egg extracts detects a single polypeptide band near 120 kDa (Figure 4C). The kinase activity of XNercc1 immunoprecipitated from egg extracts was lower than that of recombinant XNercc expressed in HEK293 cells, and it did not exhibit autoactivation when incubated in vitro with Mg^{2+} ATP. XNercc activity did not vary upon cycling the meiotic extract to interphase and back to mitosis by addition of Ca^{2+} . Addition of purified demembrated sperm nuclei was accompanied by an approximately twofold increase in XNercc kinase activity in two out of four experiments, with no change observed in the other two.

As expected, the immunoprecipitates prepared from *Xenopus* egg extracts also exhibit a single 120-kDa band immunoreactive with anti-N-XNercc; however, Coomassie Blue staining of the immunoprecipitate resolved by SDS-PAGE demonstrates that in addition to the XNercc polypeptide, there is specific coprecipitation of an array of polypeptides (Figure 4D). The bands indicated in Figure 3D were excised, subjected to tryptic digestion in situ followed by LC/MS/MS; this analysis identified γ -tubulin (this interaction is confirmed additionally by the use of anti- γ -tubulin-specific antibodies; Figure 4E), several components of the γ -tubulin ring complex (specifically GCP2/Xgrip109, GCP3/Xgrip110, and GCP6/Xgrip210), the major vault protein (whose abundance exceeds by far all other polypeptides in the immunoprecipitate) and HSP70. The association of XNercc with the γ TuRC was confirmed by the finding that XNercc immunoprecipitates contained γ -tubulin and inversely that an immunoprecipitate of Xgrip109 specifically coprecipitates XNercc (Figure 4E). Thus, as in mammalian cells, *Xenopus* XNercc is physically associated with γ -tubulin complexes, specifically with the γ -tubulin ring complex, which is known to have a central role in the control of microtubule nucleation.

Immunocytochemistry was used to localize XNercc on spindles assembled in *Xenopus* egg extracts. The anti-N-XNercc antibody specifically stains the spindle poles of astral spindles (with centrosomes) (Figure 5). In contrast, no XNercc immunoreactivity is observed on the anastral spindle poles assembled either with DNA-attached beads or Ran-GTP induced asters (our unpublished data). It should be emphasized that the N-XNercc antibody shows no reactivity on immunoblot with γ -tubulin or with any component of the γ -TuRC; thus, its ability to stain the spindle pole reinforces the conclusion that the N-XNercc antibody iden-

A

Protein Kinase

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XNercc : MSALCFDTHCHDSINSDFCSVRSCC-----PQSELHYIPIDVLCHGAYCEATLYERTEDDSLUVWKEVCHLRLSEKEREDALNEIVILLGLCHDM : 82
hNercc : MSVLGEMTHCHDSINSDFCSVRSGCCCDSSPGPSASQGPFRAGGAAHQSELHYIPIDVLCHGAYCEATLYERTEDDSLUVWKEVCHLRLSEKEREDALNEIVILLGLCHDM : 110

XNercc : IIAAYNHFLDSENTLLILEYCHNGNLFDKIURQACLFQREKTVLWYLFQIVSAVSCIHGAGILHRDIKTLNIFLTKANLIKLG DYGLAGCLSEYSMARTVGTLYTMSF : 202
hNercc : IIAAYNHFMDSNTLLILEYCHNGNLFDKIURQACLFQREKTVLWYLFQIVSAVSCIHGAGILHRDIKTLNIFLTKANLIKLG DYGLAGCLSEYSMARTVGTLYTMSF : 220

XNercc : EICQGVKTSFKSDIWAAGCVLYELLTLRTFDATNPLNLCVKIVQGNHVAFLDNTYTOELIEVVAHCLSDPEKPTADEILRPILSWREEDMEKVSMLNRSNCKPE : 312
hNercc : ELCQGVKTSFKSDIWAAGCVLYELLTLRTFDATNPLNLCVKIVQGNHVAFLDNTYTOELIEVVAHCLSDPEKPTADEILRPILSWREEDMEKVSMLNRSNCKPE : 330

XNercc : TCTVTRAPIAVVTSRSSEVYVWGGGKTTTPQKLDVPIGCGWPARQVCAGDAHPAVUTVEKELYTWVNMGGGSKLHGQLGHGDRASYRQPPHVEKLGCKSVQKVSQCSDFTVG : 422
hNercc : SSTVTRAPIAVVTSRTSEVYVWGGGKSTTPQKLDVPIGCGSARQVCAGDTHFAVUTVEKELYTWVNMGGGTLHGQLGHGDRASYRQPPHVEKLGCKRAHQVSCGDDFTVG : 440

XNercc : ITDEGQLTFCGSDYYGCLGVNQSAQSEVLEPLLVDFFLNRPVDVSCGDSHIALTRSSVYTSWGCCEYGRGLGDSDDVYSPQKVSQVQCLGCIWVCCGSDGSLTLTL : 532
hNercc : VTDEGQLTFCGSDYYGCMGVDFVACPEVLEFPMGLNFFLNPNPVRQVSCGDHIVVLTNRSEVYTSWGCCEYGRGLGDSDDVYTPQKVSQVQCLGCIWVCCGSDGTFLLTL : 550

RCC1

XNercc : GKVLACGLNEFNKLGGLNQYTAQIINHEAFQVFPYTTSLTLAKQLSFYKIRISIPGRTHAAIDERGRLLTFCGSKKCGQLGVGVYERPLGINLLGGLGKQVIRVSCGD : 642
hNercc : GKVLACGLNEFNKLGGLNQYCHSGLINHEAYRQVFPYTTSLTLAKQLSFYKIRITLPGKTHAAIDERGRLLTFCGSKKCGQLGVGVYERPLGINLLGGLGKQVIRVSCGD : 660

XNercc : FTTAATADNHIFAWNGGNGELAMTPNERPGSDICTSWPRPIFGSLHRYTDLSCRGWHTILIVEKVLNSKTIKRSNSSLGSLICTLAQSSCGGSGS----PREDSDRESL : 748
hNercc : FTTAATADNHIFAWNGGNGELAMTPTERPGSDICTSWPRPIFGSLHRYTDLSCRGWHTILIVEKVLNSKTIKRSNSSLGSLICTVFPSSSPGCCCGGGGGRREDSDQRESL : 770

XNercc : TSDPSDFGFGTIEAP-ETCPFNTTENE-----SSSCPFWLQLELHARFIPMPDTPNFVSAKSSNGT-----TSMQDVRETTPDALRKP----- : 829
hNercc : TSDPSDFGFGTIEADRCMGLISPTFANGNSNGASSSCPFWLQLELHARFIPMPDTPNFVSAKSSNGT-----TSMQDVRETTPDALRKP----- : 880

Coiled coil

XNercc : -----SFOACTCSILQSEVRKLDLVSTYRHEQLLCPKRSNLALEVRQLNGCLQSAHQHNQVVNMHGEAHHQIKQLSLHWSSPPSSGNPHS-PREDSSAKRWQPLGT : 933
hNercc : KGTPLEPPACASSLSLEVRKLDLVSTYRHEQLLCPKRSNLALEVRQLNGCLQSAHQHNQVVNMHGEAHHQIKQLSLHWSSPPSSGNPHS-PREDSSAKRWQPLGT : 972

XNercc : EACRSSSGTPM : 944
hNercc : DSCRPSEL---- : 979

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B **E** **C** **D**

Figure 4. Identification and characterization of the *X. laevis* Nercc1 orthologue, XNercc. (A) Protein sequence alignment of *X. laevis* Nercc1 (XNercc) versus human Nercc1 (hNercc). (B) Recombinant XNercc autoactivates in vitro. FLAG-XNercc immunoprecipitated from transfected HEK293 cells was incubated with phosphorylation buffer containing [γ - 32 P]ATP (100 μ M) and the model substrate MBP. At the times indicated, aliquots were removed, subjected to SDS-PAGE. Coomassie stain of the FLAG-XNercc (top) and 32 P autoradiography of the

Figure 5. Immunolocalization of XNercc in mitotic spindles assembled in vitro from *Xenopus* egg extracts. Mitotic spindles were assembled in vitro from *Xenopus* egg extracts as described in *Materials and Methods*. Aliquots were stained either with anti-XNercc antibody (bottom) or normal IgG (NIgG, top). Microtubules are visualized with rhodamine-labeled tubulin and DNA with DAPI. A magnified example of a bipolar spindle showing XNercc polypeptide localization to the poles is shown (bottom). Bar, 10 μ m.

ties a pool of XNercc1 associated with the γ -tubulin and the γ -TuRC. The activation state of this spindle pole-associated fraction of XNercc1 awaits the generation of a XNercc1Thr192P (equivalent to human Nercc1Thr210) antibody.

Effects of XNercc Immunodepletion on Spindle Formation

To determine whether XNercc is necessary for the correct formation of spindles, we examined spindle formation after

the immunodepletion of endogenous XNercc1 from meiotic extracts using the anti-N-XNercc antibody. In a later series of experiments, we also used a second anti-XNercc antibody, generated against a synthetic peptide corresponding to XNercc amino acids 810–825 (henceforth referred to as anti-C-XNercc). After three rounds of immunoprecipitation, >90% of the XNercc is consistently removed from the extracts using either antibody (Figure 6A). XNercc removal does not affect the ability of the extracts to cycle through mitosis; in two experiments we observed that the generation of H1 kinase activity upon readdition of Ca^{2+} to mitotic extracts was unaffected by the prior depletion of XNercc; in addition, the chromatin remained comparably condensed in the mock depleted and XNercc-depleted extracts (our unpublished data). Moreover depletion of $\geq 90\%$ of XNercc1 did not produce an appreciable decrease ($\leq 10\%$) in the γ -tubulin content of the residual extract (Figure 6A, right) and the demembrated sperm nuclei used in the spindle assembly reactions did not contain XNercc. In the IgG mock-depleted extracts, most of the structures were bipolar spindles with condensed DNA well aligned in the midzone (Figure 6B, control immunodepletion). XNercc immunodepletion with either the N-XNercc or C-XNercc antibodies interfered markedly, both with the formation of bipolar spindles and with the alignment of the chromosomes. Fewer bipolar spindles were observed at early point times (30 min

Figure 4 (cont). FLAG-XNercc (middle) and MBP (bottom) are shown. (C) Immunoblot of a *Xenopus* mitotic egg extract for XNercc polypeptide. The affinity purified anti-XNercc antibody was used. (D) Identification of *Xenopus* egg proteins that coprecipitate with endogenous XNercc. Immunoprecipitates prepared from mitotic *Xenopus* egg extracts using normal rabbit IgG (NIgG) or anti-XNercc (a-XNercc) were washed repeatedly and subjected to SDS-PAGE. After Coomassie staining, the numbered bands were excised, digested with trypsin, and analyzed by LC/MS/MS; the polypeptides identified in each band were 1, C6/Xgrip210; 2, XNercc; 3, major vault protein; 4, GCP3/Xgrip110 and GCP2/Xgrip109; 5, HSP70; and 6, γ -tubulin. (E) XNercc coprecipitates with the γ TuRC. Left, immunoprecipitates prepared from mitotic egg extracts using normal IgG (NIgG) or anti-XNercc were subjected to immunoblot using anti-XNercc (top) and anti- γ -tubulin (bottom). Right, Xgrip109 immunoprecipitates (top) contain XNercc (middle) and γ -tubulin (bottom).

A

B

C

Figure 6. XNercc immunodepletion from mitotic egg extracts causes delayed and aberrant spindle assembly. (A) Anti-XNercc immunoblot of *Xenopus* egg extracts after three cycles of immunoprecipitation with either normal IgG or two different anti-XNercc antibodies. After three rounds of immunoprecipitation with normal IgG (NlgG), C-XNercc antibody (C-X), and N-XNercc antibody (N-X), the residual extract was subjected to immunoblot with N-XNercc antibody. Right, immunoblot for γ -tubulin of extracts subjected to immunodepletion using normal

Table 1. Effect of XNercc depletion on spindle formation in cycled egg extracts

Minutes after calcium addition	Spindle type and number	Control ID	XNercc ID
30	Monopoles	7	20
	Bipoles	6	0
40	Monopoles	2	13
	Bipoles	14	2 (2)
50	Monopoles	8	20
	Bipoles	26	4 (3)
70	Monopoles	8	16
	Bipoles	41	16

Quantification of sperm nuclei-induced monopolar and bipolar spindles in cycled egg extracts. Mitotic egg extracts were prepared, and depleted of XNercc by three rounds of immunoprecipitation with anti-N-XNercc antibodies; control extracts were treated in parallel with identical amounts of normal rabbit IgG. After addition of demembrated sperm nuclei, the extracts were cycled once by addition of calcium. Monopolar and bipolar spindles present in 2 μ l of extract were visualized at the times indicated using rhodamine-labeled tubulin and DAPI, quantified, and classified according to their morphology. Numbers in parentheses represent bipolar structures with the DNA not correctly aligned in the midzone. Control ID, extract after three rounds of immunoprecipitation with normal IgG; N-XNercc ID, extract after three rounds of immunoprecipitation with anti-N-XNercc antibody. The results from a representative experiment are shown; two other experiments gave essentially identical results.

after reentry into mitosis; Table 1 for the time course of a typical experiment), and a much higher percentage of monopoles were observed after 40 min (Figure 6B, N-XNercc immunodepletion). The latter structures were associated with DNA and contained a single spindle pole that stained with spindle pole markers such as NUMA (our unpublished data). In the average of three independent experiments, mock-depleted extracts assembled $23 \pm 6\%$ monopoles and $77 \pm 6\%$ bipolar spindles, whereas N-XNercc immunodepleted extracts $61 \pm 8\%$ monopoles and $39 \pm 8\%$ bipolar spindles, whereas a typical C-XNercc immunodepletion shows 69% monopoles and 31% bipolar spindles. Thus, both antibodies effectively deplete XNercc and produce a very similar interference with the assembly of a bipolar spindle. To confirm the specific contribution of XNercc deficiency to the impaired spindle assembly induced by repeated depletions with anti-XNercc IgG, the ability of recombinant XNercc to overcome the abnormal spindle assembly was examined. FLAG-XNercc, transiently expressed in 293 cells and affinity purified using anti-FLAG antibodies, was added

to extracts previously treated with anti-XNercc IgG. In the two experiments performed, shown in Figure 6C, addition of purified XNercc fully corrected the faulty spindle formation induced by anti-XNercc IgG. Thus, the XNercc kinase itself is necessary for proper spindle assembly in *Xenopus* egg extract spindles.

Effects of XNercc Immunodepletion on Ran-induced Aster Formation

The results described thus far indicate an important role of XNercc in the formation of a bipolar spindle; moreover, the specific association of NERCC1 with γ -tubulin and the γ -TuRC, the localization of activated NERCC1 to the spindle poles of mammalian cells, and of XNercc1 to the poles of spindles formed in vitro, suggests strongly that NERCC1 functions in spindle assembly are intimately tied in with its centrosomal localization. However, NERCC1 has been shown to bind Ran (Roig *et al.*, 2002), a small G protein thought to play an essential role in astral spindle assembly or chromatin driven microtubule assembly (Carazo-Salas *et al.*, 1999). Briefly, the exclusive chromosomal association of active RCC1, the Ran guanyl nucleotide exchange factor, creates a gradient of active RanGTP centered around the chromosomes, which enables the release of spindle forming polypeptides from inhibitory complexes (Gruss *et al.*, 2001, Nachury *et al.*, 2001). Because spindle assembly in *Xenopus* egg extracts occurs without centrosomes (Heald *et al.*, 1996), we sought to determine whether XNercc immunodepletion also affected Ran-dependent microtubule assembly. The addition of a constitutively active form of Ran, RanQ69L, loaded with GTP to CSF-arrested extracts induces the assembly of microtubule asters, mimicking chromatin-driven microtubule assembly. XNercc immunodepletion with either the N-XNercc and C-XNercc antibodies interfered with Ran-GTP-induced spindle pole formation. XNercc immunodepletion severely retarded the appearance of aster structures in response to RanGTP at early time points after Ran addition (5–15 min; Figure 7A); after 20 min, the number of structures observed in XNercc-depleted extracts approaches control values. Nevertheless, the Ran asters formed at these later times in XNercc-immunodepleted extracts show an abnormal size (Figure 7B); in contrast to the numerous long microtubule asters seen in the mock-depleted extracts, Ran-induced poles formed in XNercc-depleted extracts exhibit short microtubules emanating from a dense center. Thus XNercc1 is important to microtubule assembly through both the centrosomal and chromosomal pathways of spindle formation.

DISCUSSION

NERCC1 is a NIMA-family protein kinase that autoactivates in vitro and is activated in vivo during mitosis. NERCC1 was identified by its ability to bind another NIMA-like protein kinase, Nek6, and is capable of phosphorylating and activating Nek6 (and Nek7) in vitro and probably during mitosis in vivo. Microinjection of cultured mammalian cells with anti-NERCC1 antibodies during prophase interrupts mitotic progression, as does depletion or interference with Nek6. We have sought to gain greater insight into the mechanisms underlying NERCC1 activation in vivo and into its role in mitotic progression. The present work extends our understanding of NERCC1 in several ways. For NERCC1 activation, we use mass spectrometry and an anti-phosphopeptide-specific antibody to establish that the in vitro autoactivation of human NERCC1 is accompanied by phosphorylation at two sites on the NERCC1 activation loop; mutagenesis indicates

Figure 6 (cont). IgG or anti-N-XNercc antibodies. (B) Representative spindle structures formed in control and XNercc-depleted extracts. Mitotic egg extracts were immunodepleted using the three IgG preparations described in A. After addition of demembrated sperm nuclei, the extracts were cycled once by calcium addition. Monopoles and spindles were visualized with rhodamine-labeled tubulin and DAPI after 40 min. Control ID, extract after three rounds of immunoprecipitation with normal rabbit IgG; N-XNercc ID, extract after three rounds of immunoprecipitation with N-XNercc antibody. Bar, 10 μ m. (C) Addition of purified recombinant XNercc corrects the defective spindle assembly of XNercc-depleted egg extracts. The results of two experiments are shown; spindles were enumerated at 60–75 min after addition of sperm nuclei. AB, XNercc add back before sperm nuclei; other, multipolar structures.

A

time after Ran addition	Number of asters / field	
	Control ID	N-XNercc ID
10 min	19	0
15 min	44	2
20 min	48	26

B **Control ID** **N-XNercc ID**

Figure 7. XNercc depletion interferes with RanQ69L(GTP)-induced aster formation. *Xenopus* egg extracts were subjected to three rounds of immunoprecipitation with normal rabbit IgG (Control ID) or N-XNercc antibody (N-XNercc ID) as shown in Figure 6. At several times after addition of GTP-loaded RanQ69L rhodamine-labeled tubulin structures were quantified at low magnification. A representative time course of aster formation on the immunodepleted extracts is shown in A. (B) Structures formed in mock or XNercc-depleted extracts after 20 min of GTP-loaded RanQ69L addition. XNercc depletion, in addition to causing a delay in the appearance of tubulin assemblies, results in the appearance of smaller, more condensed structures that tend to cluster (B). Bar, 10 μ m.

that phosphorylation of at least one of the two sites identified, Thr210, is indispensable for NERCC1 autoactivation *in vitro*. Using an anti-NERCC1(Thr210P)-specific antibody, we show that the phosphorylation of this site on human NERCC1 endogenous to U2OS cells, barely detectable in exponentially growing cells, increases dramatically in cells arrested in mitosis, indicating that the NERCC1 autophosphorylation reaction characterized *in vitro* participates in the mitotic activation of NERCC1 *in vivo*. This anti-phosphopeptide antibody, which is specific for NERCC1 that has undergone activation, has enabled the delineation of several aspects of NERCC1 activation *in vivo* that were previously unappreciated.

First, it seems that mitotic activation of NERCC1 involves a very small percentage (usually <5%) of total NERCC1 polypeptides at any time. In spite of the very low fraction of NERCC1 polypeptides activated at any time during M, the sensitivity and high specificity of the anti-NERCC1(Thr210P) antibody has enabled the use of immunocytochemistry to define the subcellular localization of activated NERCC1 during mitosis. In contrast to the diffuse cytoplasmic distribu-

tion of the vast bulk of NERCC1 polypeptides in mitotic cells, the NERCC1 polypeptides that have undergone activation *in vivo* exhibit a very specific localization. Activated NERCC1 is first clearly evident during prophase, where it is highly concentrated at the centrosome, a localization that is maintained at increasing intensity up to metaphase. With the onset of anaphase, NERCC1(Thr210P) immunoreactivity at the centrosomes diminishes but becomes detectable on the chromosomes, a pattern evident through telophase. Just before disappearing, activated NERCC1 is detectable at the midbody, as two dots flanking the cytokinetic furrow. The localization of activated NERCC1 to the centrosome is entirely consistent with the physical association of NERCC1 with γ -tubulin, another polypeptide that is distributed throughout the cytoplasm but is recruited to and concentrated at the centrosome in G₂, increasing in abundance at this site through prophase. It is plausible to propose that the localization of activated NERCC1 to the centrosome is mediated by its ability to bind to γ -tubulin and to the γ -tubulin ring complex; however, this has yet to be demonstrated directly. Although γ -tubulin is relatively abundant throughout the cytoplasm in interphase,

it is recruited to the centrosome in preparation for mitosis, and a variety of proteins have been proposed to contribute to the centrosomal recruitment and anchoring of γ -tubulin; one or more of those may therefore be responsible indirectly for the centrosomal localization of Nercc1.

Regarding the role of γ -tubulin and centrosomal localization in the regulation of Nercc1 activity, several considerations are pertinent. Nercc1 can be coprecipitated with γ -tubulin from extracts of exponentially growing cells, wherein Nercc1 is largely inactive, suggesting that Nercc1 activation is not required for association with γ -tubulin. Moreover, the XNercc1 immunoprecipitated from CSF-arrested egg extracts exhibits little autoactivation *in vitro* and very low kinase activity, raising the possibility that γ -tubulin, the γ -TuRC complex or other of the proteins coprecipitating with XNercc may contribute to the suppression of Nercc1 autoactivation *in vivo* during interphase. We have no information at present as to whether Nercc1 is activated in the cytosol and subsequently recruited to and concentrated at the centrosome or activated only after its centrosomal localization. The latter possibility seems more attractive, inasmuch as Nercc1 is a good substrate for activated Cdc2, which is detectable selectively at the centrosome early in prophase (Jackman *et al.*, 2003). Thus, we propose that a fraction of Nercc1 may be recruited to the centrosome as a passenger with the γ -TuRC, and once there undergoes activation, perhaps initiated by phosphorylation by activated Cdc2 followed by autophosphorylation. The mechanism and significance of the redistribution of activated Nercc1 during and after anaphase, onto the chromosomes and into the midbody, are unknown.

Based on the dramatic slowing of electrophoretic mobility, it is likely that a majority of the activated Nercc1 polypeptides undergo a posttranslational modification other than phosphorylation. Previous work showed that autophosphorylation of Nercc1 *in vitro* results in a modest slowing of mobility on SDS-PAGE; the mobility of the Nercc1 polypeptides in extracts of mitotic U2OS cells, as visualized by anti-Nercc1 polypeptide blot, also exhibit a similar modest upshift (compared with Nercc1 in exponentially growing cells) so as to overlap the mobility of the Nercc1 activated *in vitro*. Surprisingly, although Nercc1(Thr210P) immunoblot of extracts of mitotic U2OS cells and of Nercc1 immunoprecipitates prepared from these extracts exhibit immunoreactivity coincident with the slightly upshifted Nercc1 band (near 120 kDa), the majority of immunoreactive Nercc1(Thr210P) polypeptides exhibit a markedly slower mobility on SDS-PAGE, migrating at an apparent M_r near 170 kDa. The identity of the 170-kDa anti-Thr210P band as Nercc1 is confirmed by its specific immunoprecipitation with two independent anti-Nercc1 polypeptide antibodies. The nature of the posttranslational modification responsible for this large upshift is as yet unknown; however, it is likely to reflect a covalent modification other than phosphorylation (preliminary tests suggest that it is not related to ubiquitination or ADP-ribosylation; our unpublished data). Notably, the hypershifted Nercc1(Thr210P) polypeptides represent a very small fraction of the total Nercc1 (Figure 1E), inasmuch as a band near 170 kDa is only visible in anti-Nercc1 polypeptide immunoblots of mitotic U2OS extracts or Nercc1 immunoprecipitates from such extracts after prolonged exposure. At present, we can only speculate as to the functional significance of this modification. Among the possibilities are that it may precede and be required for activation of Nercc1 catalytic function. Alternatively, it may follow activation and be involved in Nercc1 deactivation and/or degradation; although overall levels of Nercc1 are

unaltered during the cell cycle, selective degradation of activated Nercc1 could readily be overlooked. Independently of whether the Nercc1 modification is involved in activation or deactivation, it may be significant for the specific cellular localization of active Nercc1 during mitosis.

Regarding the role of Nercc1 in mitotic progression, we showed previously (Roig *et al.*, 2002) that interfering with Nercc1 during prophase by microinjection of antibodies leads to prometaphase arrest or defective mitosis resulting in aneuploidy. The present results establish that Nercc1 plays a specific and significant role in spindle assembly. Depletion of *Xenopus* Nercc1 from meiotic egg extracts interferes with the assembly of bipolar spindles, and this is reversed by addition of purified recombinant XNercc; moreover, XNercc depletion also interferes with the ability of Ran-GTP to promote the coalescence of spindle-pole-like structures. In each case, an 80–90% depletion of XNercc causes a marked delay in these assembly processes, suggesting that XNercc deficiency interferes with the availability or functionality of one or more components critical for microtubule assembly. Ran-GTP induction of asters is dependent on the release of TPX2 and NuMA from inhibitory importin complexes. Interestingly, preliminary experiments indicate that XNercc can bind and phosphorylate recombinant TPX2; the significance of this interaction is currently unclear, inasmuch as TPX2 immunoprecipitates do contain detectable XNercc. The significance of the strong association of XNercc with the vault complex, an abundant RNA protein structure of unknown function, is also unknown.

In conclusion, the present work demonstrates that the mitotic kinase Nercc1 is specifically associated with γ -tubulin and the γ -TuRC. Moreover, activated Nercc1 is localized in the centrosomes early in mitosis and later migrates to the chromosomes and into the cytokinetic furrow. Depletion of Nercc1 from *Xenopus* egg extracts does not impair mitotic cycling, but it interferes strongly with the ability of sperm nuclei to promote assembly of bipolar spindles and of Ran-GTP to nucleate aster-like assemblies of microtubules; thus, one important function of Nercc1 is in the regulation of the assembly of mitotic spindle. The molecular basis for this action is currently under investigation. Thus, like Nek2, Nercc1 is involved in the control of the cellular microtubule machinery, and the two metazoan NIMA-like protein kinases, activated at different phases of the cell cycle, together execute at least a subset of the cell cycle functions performed by the ancestral NIMA protein kinase.

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